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Death of *Bacillus subtilis* Auxotrophs Due to Deprivation of Thymine, Tryptophan, or Uracil

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ABSTRACT

PRITIKIN, WILLIAM B. (University of California, Los Angeles), AND W. R. ROMIG. Death of *Bacillus subtilis* auxotrophs due to deprivation of thymine, tryptophan, or uracil, J. Bacteriol. **92**:291–296. 1966.—Auxotrophic mutants of *Bacillus subtilis* 168 that require either tryptophan, uracil, or thymine died rapidly when deprived of any of these compounds. Phage PBS1 was produced by infected *B. subtilis* 168 (*thy try-2*) deprived of thymine. Phage PBS1 was not produced by infected *B. subtilis* 168 (*try-2*) deprived of tryptophan or infected *B. subtilis* 168-15 (*try-2 ura*) deprived of uracil. *B. subtilis* 168 *thy try-2* and 168-15 could be transduced by phage PBS1 after prolonged deprivation of tryptophan or uracil, respectively. When *B. subtilis* 168-15 was transduced to uracil independence by phage PBS1, the uracil-independent transductants became immune to uracil-less death within 10 min of exposure to phage, and began to multiply within 2 hr after exposure to phage at an incubation temperature of 46 C.

The cultural behavior of thymine-requiring mutants of *Escherichia coli* (2, 11), *Bacillus subtilis* ATCC 6633 (W. J. Brabander, Ph.D. Thesis, Univ. California, Los Angeles, 1963), and *B. megaterium* (19) is similar. When these mutants are deprived of thymine in an otherwise complete medium, the colony-forming ability declines at an easily observable rate. This phenomenon is called "thymineless death." At 37 C, the decline becomes apparent within 1 or 2 hr. There may or may not be an increase in colony count before the decline, depending on cultural conditions before thymine deprivation. With double mutants, requiring thymine and one amino acid, simultaneous deprivation of thymine and the amino acid causes no reduction in viability, or causes a reduction which is slight compared to that caused by the deprivation of thymine alone. Deprivation of only the required amino acid causes no loss of colony-forming ability with starvation periods of 2 to 3 hr in *E. coli* or *B. subtilis* ATCC 6633; however, 2 hr of histidine starvation causes a decline in the viability of a *B. megaterium* mutant (19). Two hours of uracil starvation of a *B. subtilis* ATCC 6633 mutant causes no decrease in viability (Brabander, Ph.D. Thesis). Recently, mutants of the transforming strain *B. subtilis* 168 (14), which require uracil (isolated by Jacques Pene) and thymine (4), have become available. We report here on further cultural and genetic studies on these two mutants and the parent strain, 168.

Nester and Stocker (9) found that markers transformed into *B. subtilis* are not expressed until 3 or 4 hr after competent cells are exposed to transforming deoxyribonucleic acid (DNA). It would be difficult to determine the time required for the expression of a transduced marker by measuring enzyme production, because the number of recombinants produced by transduction is so small. A method is described here for estimating the time required for expression of a transduced marker.

MATERIALS AND METHODS

Media. Enriched TY (ETY) broth: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g; $FeCl_3 \cdot 6H_20$, 0.25 mg; $CaCl_2$, 0.1 g; $MnCl_2$, 1.26 mg; distilled water, 1,000 ml. Minimal medium: $(NH_4)\ SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate $\cdot 5\frac{1}{2}H_2O$, 1 g; L-glutamic acid, 1 g; glucose, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.25 mg; $CaCl_2$, 0.1 g; $MnCl_2$, 1.26 mg; distilled water, 1,000 ml. For solid media, Difco agar was added in the following amounts: for colony counts, 20 g per liter; for phage assay base plates, 15 g per liter; for phage assay overlay, 6 g per liter. Supplements, when re-

quired, were added in the following amounts: thymine, 100 to 250 μg/ml; uracil, 50 μg/ml; tryptophan, 50 μg/ml. These supplements are probably present in excess (4); however, the thymine-requiring mutant grew poorly on ETY medium unless a thymine supplement was added. All supplements were Calbiochem A grade.

Bacteria. B. subtilis 168 requires (8) tryptophan (*try-2*) and can be made highly competent for transformation (14). *B. subtilis* 168-15 is an ultraviolet-induced mutant of 168 which requires uracil and tryptophan. *B. subtilis* 168 *thy try-2* (4) requires thymine and tryptophan. *B. subtilis* SP0001+ (obtained from Pierre Schaeffer) is prototrophic and asporogenous (derived from 168).

Phage. Phage PBS1 (15, 16) is a phage of *B. subtilis* capable of generalized transduction.

Crude PBS1 suspensions were prepared by sedimenting the remaining bacteria from ETY broth lysates of *B. subtilis* SP0001+ by centrifugation. Concentrated PBS1 was prepared by centrifuging the clarified ETY lysates at 47,120 × *g* for 60 min. The pelleted phages were suspended in a buffer solution containing $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4 in the same concentrations as in minimal medium. Concentrated phage suspensions used for transduction were treated with chloroform.

Purified PBS1, with enriched transducing activity, was prepared as follows. Cesium chloride was added to a concentrated phage suspension to make a solution of density 1.45 g/cm³. This CsCl phage suspension was centrifuged at 34,000 rev/min for 24 hr in a Spinco SW 39 rotor. Drops were collected by puncturing the bottom of the centrifuge tube, and were diluted with the buffer solution. The diluted drops in the region of the visible phage band were tested for uracil marker transducing activity (18). The collection tube showing the highest uracil-transducing activity was used to make the enriched transducing phage (ETP) preparation. The tubes with high activity received material less dense than the visible phage band. The fractions with high transducing activity from three gradients were pooled to make the ETP used in the experiments reported here. The transduction frequency of the ETP, expressed as transductants/plaque-forming unit, at a multiplicity of infection (MOI; plaque-forming units/colony-forming units) of 0.14, was, for the uracil marker, 7.8×10^{-5}, and for the tryptophan marker, 4.3×10^{-5} (experiment reported in Fig. 6). The highest transduction frequencies obtained by use of unfractionated phage suspensions were, for the uracil marker, 1.5×10^{-5} (MOI = 0.08), and for the tryptophan marker, 1.2×10^{-5} (MOI = 0.11).

Single-step growth experiments. The method described by Adams (1) was used. Before infection, *B. subtilis* 168 *thy try-2* was deprived of thymine for 100 min, *B. subtilis* 168-15 was deprived of uracil for 60 min, and *B subtilis* 168 was deprived of tryptophan for 50 min. The incubation temperature during deprivation was 46 C, and air was bubbled through the medium. After the initial starvation period, the bacteria were transferred by centrifugation to fresh minimal medium of the same composition, to minimize the possible metabolism of compounds leaked by cells. Phage was then added.

Transduction experiments. Transduction of uracil-starved *B. subtilis* 168-15 was performed as follows. The bacteria were grown at 46 C in minimal medium with tryptophan and uracil. In this and all subsequent incubations, air was bubbled through the medium. The bacteria were then transferred by centrifugation (at 4 C) to minimal medium with tryptophan and incubated at 46 C for 50 min. The bacteria were then transferred by centrifugation (4 C) to fresh minimal medium with tryptophan. To a portion of this culture, phage (ETP) was added. Another portion was left uninfected for control experiments. Both cultures were incubated for 10 min (to allow maximal phage adsorption in the infected culture) before withdrawing the first sample. The incubation temperature was 46 C. Samples to be tested for transduction were centrifuged at 4 C and suspended in minimal medium before plating.

Transduction of *B. subtilis* 168 *thy try-2* was performed in a similar manner, except that concentrated phage was used, and the bacteria were infected at various times after deprivation of thymine.

RESULTS

The loss of colony-forming ability of *B. subtilis* 168 *thy try-2* due to thymine deprivation, "thymineless death," is shown in Fig. 1. The optical density of cultures deprived of thymine rose nearly as fast for up to 2 hr at 46 C as the optical density of fully supplemented cultures (Fig. 2). Thymine-starved cultures contained many long, nonseptate, nonmotile cells. These results are typical of thymine-requiring bacteria deprived of thymine(2, 19; Brabander, Ph.D. Thesis). *B. subtilis* 168 *thy try-2* could be transduced to thymine independence after 90 min of thymine starvation.

Single-step growth experiments with thymine-starved *B. subtilis* 168 *thy try-2* infected with PBS1 indicated that PBS1 could grow in these thymine-starved cells. Concentrated phage suspensions were used in these experiments, and

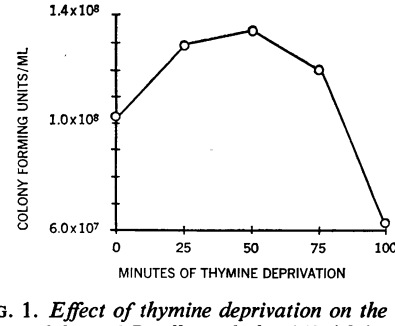

FIG. 1. *Effect of thymine deprivation on the colony-forming ability of Bacillus subtilis 168 (thy). The incubation temperature was 46 C.*

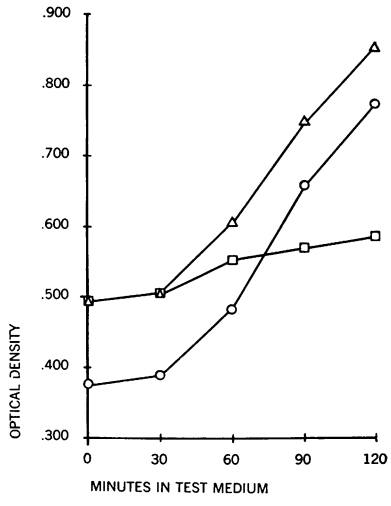

FIG. 2. *Optical density of cultures of Bacillus subtilis 168 (thy) in minimal medium supplemented as follows: ○, tryptophan and thymine; △, tryptophan; □, no supplement.*

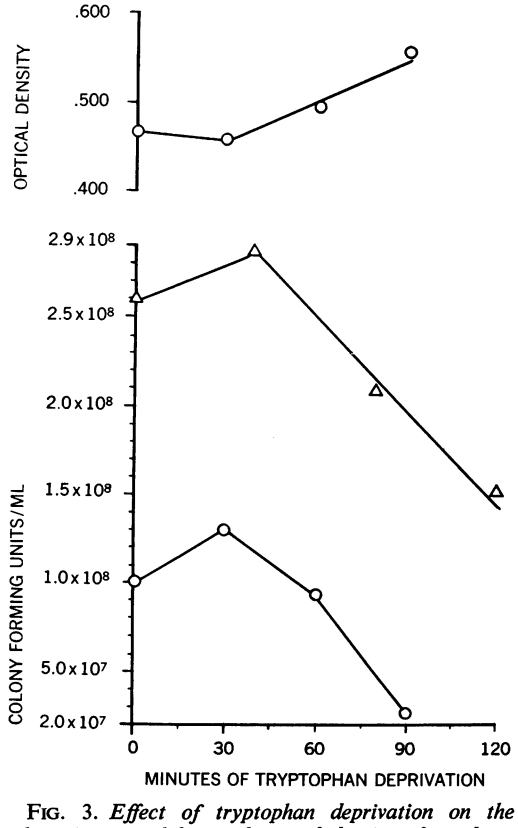

FIG. 3. *Effect of tryptophan deprivation on the colony-forming ability and optical density of a culture of Bacillus subtilis 168. (○) Incubation temperature, 46 C; (△) incubation temperature, 37 C.*

were diluted 10^{-2} in the adsorption mixture to minimize the possibility of thymine contamination of the culture.

Depriving cultures of *B. subtilis* 168 of tryptophan caused a loss of colony-forming ability in the culture. "Tryptophanless death" was readily observed during relatively short incubation periods at 46 C or 37 C (Fig. 3). The optical density of the cultures rose only a little during the period of observation. During the entire period of observation, most cells retained their motility and appeared to be normal except for some enlargement (but not elongation) late in the observation period.

Single-step growth experiments did not show any net phage production by cells starved of tryptophan for 50 min before phage infection.

Depriving *B. subtilis* 168-15 of uracil caused a decrease in colony-forming ability of the culture (Fig. 4). The optical density of the culture rose slightly during the period of observation. For up to 60 min of uracil starvation, the cells appeared to be normal and motile, but at 90 and 120 min many nonmotile, kinked or curved cells were observed. Single-step growth experiments showed no net phage production by cultures starved of uracil for 60 min before phage infection.

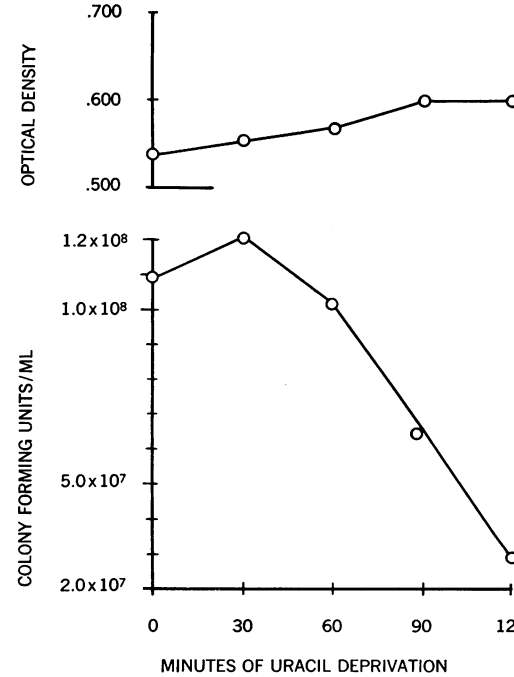

FIG. 4. *Effect of uracil deprivation on the colony-forming ability and optical density of Bacillus subtilis 168-15. The incubation temperature was 46 C.*

When a culture of 168-15 starved of uracil was transferred (by centrifugation at 4 C) to fresh minimal medium with tryptophan, a reduction in the optical density of the culture was observed (Fig. 5). The initial optical density decrease was greater in the cultures infected for transduction. In uninfected cultures the colony count continued to decrease, roughly paralleling the optical density drop.

The behavior of uracil-independent and tryptophan-independent transductants is shown in Fig. 6. The number of uracil-independent transductants remained constant for 1 hr (after an initial 10-min period to allow for phage adsorption), and by 2 hr they had begun to multiply. The tryptophan-independent transductants died rapidly. In the experiment shown in Fig. 6, the tryptophan-independent transductants were concentrated by centrifugation. Ordinarily, the number of tryptophan-independent transductants was about 50% of the number of uracil-independent transductants.

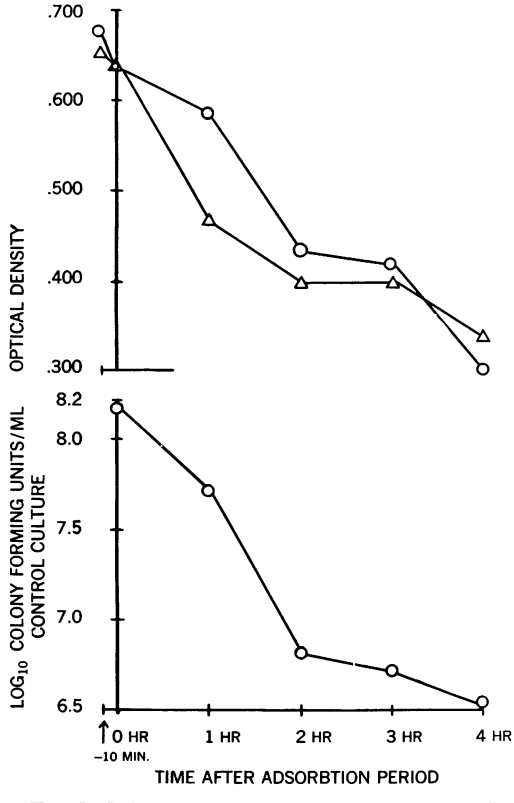

FIG. 5. *Behavior of uracil-starved cultures of Bacillus subtilis 168-15 after transfer to fresh minimal medium with tryptophan. The preliminary starvation period was 50 min. Immediately after transfer, phage (multiplicity, 0.14 plaque-forming unit/bacterium) was added to the transduction culture, and a 10-min period was allowed for phage adsorption. On the time scale, −10 min represents the time of phage addition to the transduction culture. Incubation temperature was 46 C before and after transfer. (○) Control culture (contained no phage); (△) transduction culture (phage-infected).*

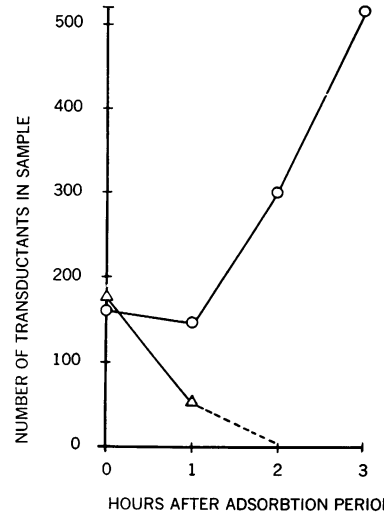

FIG. 6. *Survival of transductants in a phage-infected culture of Bacillus subtilis 168-15 deprived of uracil. "ETP" phage preparation used; MOI = 0.14. (○) Uracil-independent transductants; (△) tryptophan-independent transductants.*

DISCUSSION

ETY broth supplemented with thymine was chosen as a growth medium for *B. subtilis* 168 *thy try-2* because it supports a rapid growth rate and a high colony count/optical density ratio; however, the time required for onset of thymineless death was variable when ETY was used to grow the cells.

Phage PBS1 has been reported to contain deoxyuridine in its DNA instead of thymidine (17). The results of the single-step growth experiments with PBS1-infected, thymine-starved *B. subtilis* 168 *thy try-2* suggest that thymine from the host cell is not required for the growth of PBS1.

Death of *B. subtilis* 168 and 168-15, when starved of uracil at 46 C, occurs much more rapidly than the death of mutants of *B. subtilis* ATCC 6633 (Brabander, Ph.D. Thesis), *E. coli* (2, 8), or *B. megaterium* (19) when deprived of uracil, adenine, or various amino acids, at 37 C. The *E. coli* and *B. subtilis* ATCC 6633 mutants show little or no loss of viability under these conditions for the usual observation periods of 2 to 3 hr. The death of *B. subtilis* 168 or 168-15 is not due to the (46 C) incubation temperature (Fig. 3).

B. subtilis SB19 (derived from 168) will grow at 51 C in TY broth medium (10). In the experiments reported here, the 46 C temperature was used because it increased the growth rate of cultures in minimal medium. The average doubling time of *B. subtilis* 168 in minimal-tryptophan medium at 37 C was 30 min; at 46 C, it was 23 min.

The death of *B. subtilis* 168 and 168-15 due to tryptophan or uracil deprivation is probably related to the general sensitivity of vegetative cells of *B. subtilis* to any conditions which cause a cessation of growth. We have confirmed that deprivation of oxygen or carbon source will cause rapid death and lysis of the cells. *B. subtilis* held at ice bath temperatures may lyse (13). We found that centrifugation at 4 C (duration about 15 min), or holding at ice bath temperatures for up to 10 min, or both, did not kill growing *B. subtilis* 168, or 168-15, or uracil-independent transductants of uracil-starved 168-15. The cold centrifugation procedure may have something to do with the lysis of uracil-starved 168-15 noted in Fig. 5 (compare with Fig. 4).

In the transduction experiments, transduction occurred under conditions where there was presumably no net DNA synthesis at the culture level. However, DNA synthesis by individual cells cannot be excluded. Ribonucleic acid, DNA, and protein can be found in cell-free supernatant fluids of growing cultures of *B. subtilis* (3). It is possible that such compounds are present in starved, but unlysed, cultures as well, and that they might be metabolized by some cells. In Fig. 6, it is shown that tryptophan-independent transductants of *B. subtilis* 168-15 undergo uracil-less death in a medium lacking uracil, whereas uracil-independent transductants do not. If metabolism of compounds excreted into the medium, or if DNA injected by the transducing phage, allows the survival of uracil-independent transductants of uracil-starved cells, it does not do the same for tryptophan-independent transductants of uracil-starved 168-15, even though there is tryptophan in the transduction medium. The results shown in Fig. 6 also indicate that the transductants are not selected from a special class of cells resistant to uracil-less death before addition of phage.

In transformation of *B. subtilis*, donor DNA is integrated into the recipient chromosome within 10 min (12), whereas expression of the marker is not detectable for 3 or 4 hr after integration (9). Presumably, the lag in expression is due to metabolic dormancy of competent cells (4, 9). Our results indicate that noncompetent cells express a transduced uracil marker within 10 min, since uracil-independent transductants become immune to uracil-less death within this time (Fig. 6). The lag before the multiplication of the transductants (Fig. 6) probably represents the time required for synthesis of the compounds lost during starvation, plus the time required for division. The lag before multiplication of transductants may represent in part the time required for integration of the marker. The bacterial DNA in phage PBS1 is not covalently bonded to phage DNA (7), and the mechanism for integration of PBS1-transduced markers may be the same as that for transformed markers. If this were true, integration would probably comprise no more than 10 min of the lag period. However, transformants which divide but do not replicate their new marker for several generations have been reported (5).

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